J. Biosci., Vol. 21, Number 4, June 1996, pp 511-517.© Printed in India.

Prolactin augments luteinizing hormone binding to rat Leydig cells in serum-free culture

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MS received 18 November 1995; revised 8 April 1996

Abstract. Effect of prolactin on the testicular luteinizing hormone binding was studied in a serum-free culture system. By the collagenase digestion of decapsulated testes taken out from 25-day-old rats, Leydig cells were isolated and cultured for 7 days in DME/F12 (1:1) medium supplemented with insulin, transferrin, epidermal growth factor, and gentamicin. The cultured cells exhibited the 3ß-hydroxysteroid dehydrogenase activity. Hill plots constructed from the data of competition experiment showed that the dissociation constant (K_d) was 0.33×10^{-10}M. The K_d value was approximately the same as the known value for the rat testicular homogenates. When the Leydig cells were cultured with ovine prolactin for the last 3 days of 7-day culture period, the binding of luteinizing hormone increased to 1·7-fold of that in the control group. From these results it is concluded that prolactin acts to up-regulate the binding of luteinizing hormone to rat testicular Leydig cells in serum-free culture.

Keywords. Prolactin; luteinizing hormone binding; Leydig cell.

1. Introduction

Prolactin (PRL) is one of the factors regulating testicular function in mammals (Bartke 1980; Mazzocchi *et al* 1990). When PRL was administered to hypophysectomized immature (Purvis *et al* 1979) and adult (Zipf *et al* 1978) male rats, the binding of luteinizing hormone (LH) to isolated Leydig cells and testis homogenates respectively were elevated. In our previous reports (Takase *et al* 1990a, b), we also showed that the treatment of hypophysectomized adult male mice with PRL induced an augmentation of testicular LH binding per Leydig cell without significant increase in the number of Leydig cells. These results conform to the earlier report of Purvis *et al* (1979) that PRL up-regulates LH receptors in Leydig cells.

In order to understand whether the up-regulatory effect of PRL on Leydig cells is a direct one or not, the present study was undertaken in a serum-free culture system.

2. Materials and methods

2.1 *Chemicals*

NIADDK-ovine PRL-19 and highly purified NIADDK-rat LH-I-6 were provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases

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(NIADDK) Bethesda, MD, USA. NIH-LH-S-19 was supplied by the National Institutes of Health, Bethesda. Collagenase type IV was the product of Cooper Biomedical (Malvern, PA). Dulbecco's modified Eagle's medium and Ham's nutrient mixture medium (DME/F12, 1:1) in powder form, soybean trypsin inhibitor, epidermal growth factor (EGF), transferrin, insulin, and gentamicin were purchased from Sigma (St. Louis, MO).

2.2 *Preparation and culture of Leydig cells*

Testes were aseptically removed from 25-day-old male rats of the Wistar/Tw strain. For the preparation of Leydig cells, the testes, after removal of tunica albuginea, were subjected to collagenase digestion as reported previously (Takase *et al* 1988). In brief, the decapsulated testes were digested with 0·05% collagenase and 0·005% soybean trypsin inhibitor in DME/F12 medium at pH 7·4 for 15 min at room temperature. After the incubation. loosened testes were agitated by gentle pipetting with a thick pipette and sediment allowed to keep for 10 min at 4°C. Supernatant was aspirated. This procedure of pipetting, sedimentation and aspiration was repeated three times. The supernatants were pooled and centrifuged at 350 *g* for 5 min at 4°C. The resulting pellet was resuspended and cultured in DME/F12 supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), EGF (2·5 ng/ml) and gentamicin (20 μg/ml) for 7 days at 32°C in a humidified atmosphere of 95% air –5% CO_2. Initial cell concentration was $5{\cdot}0 \times 10^5$ cells per well of 24-rnultiwell culture plate (Corning) and the volume of culture medium per well was 0·5 ml. The medium was renewed daily from the second day of culture. Ovine PRL was dissolved in 0·03M $NaHCO_3$ containing 0·15 M NaCl (pH 9.5) at a concentration of 1 mg/ml, and 20 μg of ovine PRL was added to each well from the fourth day of culture.

2.3 *Staining of cultured cells*

For morphological observation, cells were placed on coverslips after 7 days of culture. fixed with 100% methanol for 10 min, and stained with Giemsa solution. For the demonstration of 3ß-hydroxysteroid dehydrogenase (HSD) activity. Leydig cells were incubated on coverslips in DME/F12 medium containing LH-S-19 (0.5 μg/ml) for 24 h. After incubation, cells were then added to the incubation mixture containing nicotinamide adenine dinucleotide (0·54 mM), nitroblue tetrazolium (0·16 mM), nicotinamide (4 mM), and dehydroepiandrosterone (1 mM) in 10 mM phosphate- buffered saline (pH 7·4) at 37°C for 1 h (Levy *et al* 1959). These chemicals were the products of Sigma.

2.4 *Binding assay of LH*

At the end of culture, Leydig cells were scraped out from the culture dishes with rubber policeman into 0·04 M Tris-HCl buffer (pH 7·4) containing 0·005 M $MgSO_4$ and 0·1% bovine serum albumin, and collected by centrifugation at 11,000 *g* for 10 min at 4°C. A rubber policeman is the instrument, which consists of a lump of rubber fixed to the tip of a glass stick, for scraping out cells cultured on the culture dishes. Immediately after centrifugation, pellets were frozen on dry ice in ethanol and stored at — 80°C until binding assay was carried out. Highly purified rat LH (NIADDK-rLH-I-6) was iodinated with ^{131}I- by the lactoperoxidase method (Tsutsui and Ishii 1980; Tsutsui

1991). The specific activity of [^{131}I] LH was 2·56 M Bq/μg. For the binding assay, the stored cells were thawed and homogenized in cold Tris-HCl buffer. The homogenates were centrifuged at 11,000 *g* for 20 min at 4°C. The resulting pellets (particulate fraction) were resuspended in cold buffer and adjusted to 15 or 12·5 μl of the buffer per well sample for the competition curve or the PRL experiments, respectively. The particulate fraction (50μl) and 25 μl of [^{131}I] LH (0·097 ng) were incubated with or without excess dose of unlabelled NIH-LH-S-19 (l·5 μg, 25 μl) for nonspecific or total binding, respectively. Total volume of reaction mixture was adjusted to 100 μl by adding 0·04 M Tris-HCl buffer (pH 7·4). Specific binding was calculated by subtracting nonspecific binding from the total binding. For drawing competition curve, various doses of unlabelled NIADDK-rLH-I-6 were used as competitors.

2.5 *Radioimmunoassay of testosterone*

After 7 days of culture, 0·1 μg of ovine LH (NIA DDK-oLH-25) was added to each well and Leydig cells were further cultured for 3, 6, 12 or 24 h. After the cultivation,

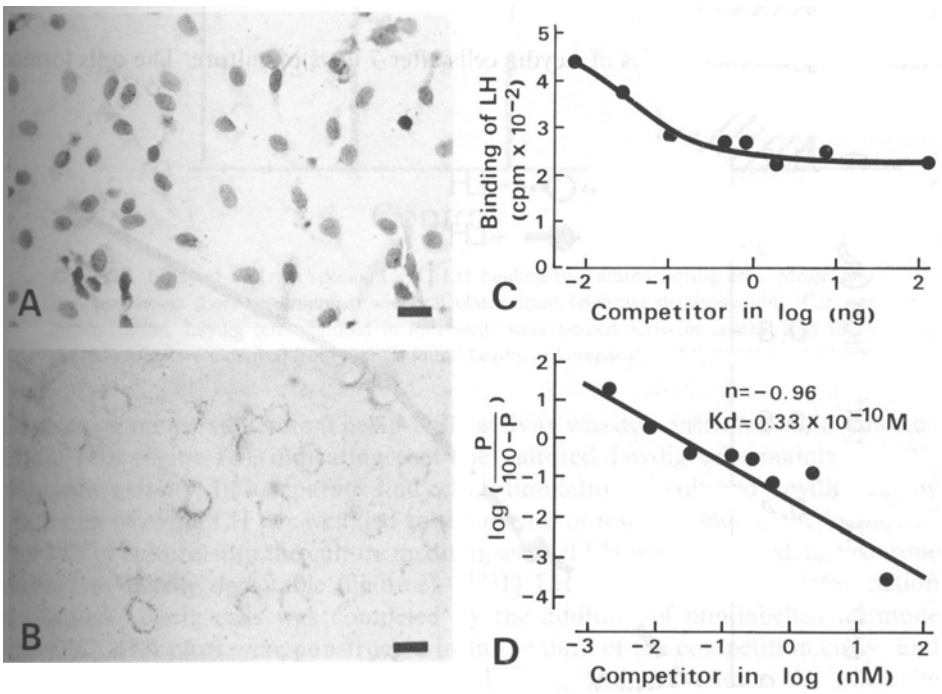

Figure 1. Basic characteristics of rat Leydig cells cultured for 7 days in a serum-free medium. (**A**) Leydig cells stained with Giemsa solution. Note almost homogeneous population. Bar: 20 μm. (**B**) Leydig cells stained for 3ß-hydroxysteroid dehydrogenase activity. Reaction products as intracytoplasmic deposits. Bar: 20 μm. (**C**) Competition curve for LH binding to Leydig cells cultured for 7 days. Twenty-seven wells were pooled. Each point depicts the mean of duplicate determinations. (**D**) Hill plots constructed from the data of competition curve. Hill coefficient (*n*) was determined from the slope of fitted line of the plots. On the Hill plots, the value on the abscissa corresponding to 0 on the ordinate represents dissociation constant (K_d). *P* indicates per cent of specific LH binding to total binding.

the spent medium was collected. Testosterone in the medium was extracted with 2 ml of ether three times and the concentrations were measured by radioimmunoassay using a double antibody method according to our routine methods (Tsutsui 1991). The antisera against testosterone-11-succ-BSA (Teikoku Zoki Pharmaceutical Co., Ltd., Tokyo, Japan) and the [1,2,6,7-^{3}H] testosterone (Radiochemical Center) were used in the radioimmunoassay of testosterone. Separation of bound and free hormones was performed by the centrifugation (l,800*g* for 20 min at 4°C) after reaction with second antibody. The cross-reaction of the antiserum against 5α-dihydrotestosterone was 15·3%. Because the assay was performed without chromatographic purification of testosterone, the calculated testosterone values may be related to the total concentrations of testosterone plus 5α-dihydrotestosterone. The testosterone concentration assayed in 0·5 ml of the spent medium was expressed in terms of ng of testosterone per well.

2.6 *Statistics*

Statistical analysis was performed by Student's *t*-test.

3. Results and discussion

Figure lA shows the profiles of Leydig cells after 7 days of culture. The cells formed

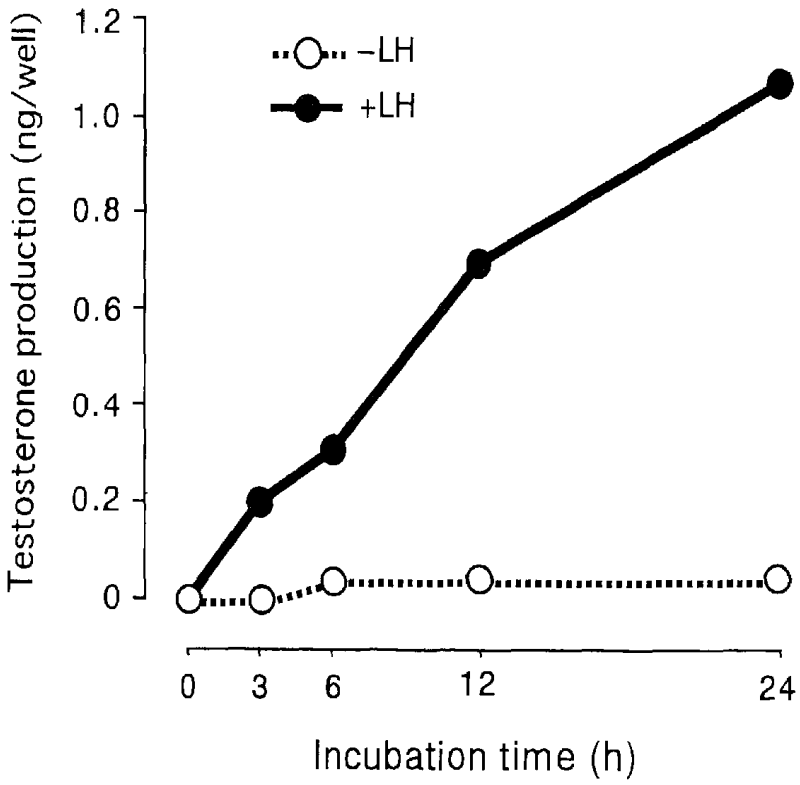

Figure 2. Testosterone production in cultured rat Leydig cells. After 7 days of culture, Leydig cells were incubated with or without 0·1 μg of ovine LH per well (0·5 ml of culture medium). Results are duplicate determinations. Testosterone concentrations were measured by radioimmunoassay.

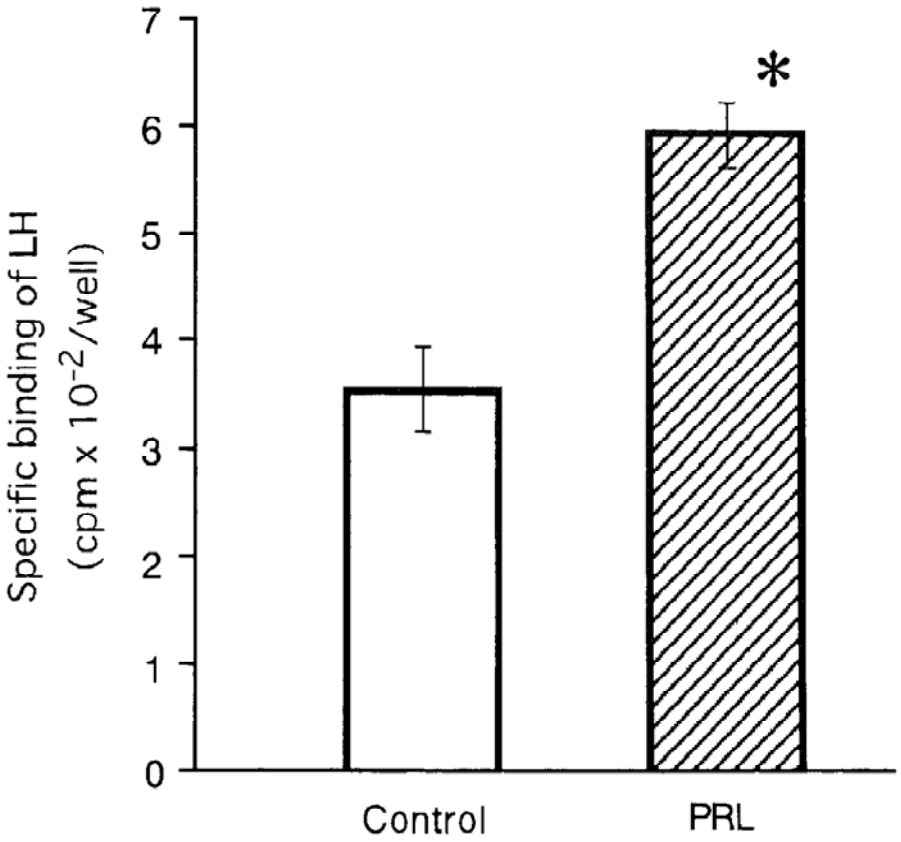

Figure 3. Effect of PRL on specific [$_{131}$I] LH binding to cultured Leydig cells. Means and standard errors (bars on columns) were calculated from triplicate determinations. For one determination, Leydig cells cultured in four wells were pooled. Cells of control and PRL-treated groups were derived from a single initial Leydig cell preparation. *P <0·05 (*vs*. control).

a monolayer on the substrate. The 3ß-HSD activity was demonstrated in the cultured Leydig cells (figure 1B), indicating that the cultured Leydig cells maintained steroidogenic activity. In a separate study, the stimulation of cultured Leydig cells by LH (0·1 μg of ovine LH per well) led to the release of testosterone (about 1·0 ng/well after 24 h of culture) into the culture medium, while if LH was not added, testosterone release was hardly detectable (figure 2). [^{131}I] LH binding to particulate fraction of cultured Leydig cells was competed by the addition of non-labelled hormone (figure lC). Hill plots were constructed from the data of the competition curve, and a significant fitted line (P < 0·05) was drawn on the Hill plots (figure 1D). Since the same hormone species as radiolabelled ligand was used as a competitor, the slope of the fitted line should be closer to — 1 on the Hill plots. In fact, the slope of the fitted line was calculated as — 0·96, and the value was not significantly different from the value of – 1. The value of K_d (0·33 × 10^{-10} M) is comparable to the value (1·25 × 10^{-10} M for hCG) reported by Wahlstrom *et al* (1983) in the rat testis homogenate. Therefore, it is considered that Leydig cells in serum-free culture maintain the *in vivo* characteristics of the cells, and afford a good model for the analysis of regulation of testicular LH receptors by PRL.

When the cultured Leydig cells were treated with PRL for 3 days, the specific LH binding increased to 1·7-fold that of control cultures (figure 3). The augmentation of LH binding by PRL treatment may be explained either by the increase in the number of Leydig cells or by the increase in the number of LH binding sites per Leydig cell. There is evidence indicating that the number of Leydig cells per testis does not change by the administration of PRL to hypophysectomized adult mice (Takase *et al* 1990a). Therefore, the possibility that an increase in the LH binding by PRL treatment was due to the increase in the number of LH binding per Leydig cell may be more feasible in the present study. In fact, no mitotic figures were encountered throughout the culture period in both control and PRL-treated groups. Moreover, it can be considered another possibility that PRL modulated the binding affinity. However, because Purvis *et al* (1979) reported that no change in an affinity of the hCG binding to dispersed Leydig cells prepared from the testes of immature hypophysectomized rats exposed to PRL was observed, in the present study, it may be excluded this possibility. Although Leydig cells were not purified before cultivation, the population was almost homogeneous (figure 1A). In addition, it was immunohistochemically determined that both PRL and LH receptors were localized only in Leydig cells among other testicular components (Wahlstrom *et al* 1983). Therefore, it is possible that PRL acted directly on Leydig cells to increase LH binding. On the other hand, it was reported that administration of PRL to cultured granulosa cells of rat (Chen *et al* 1987) or pig (Lane and Chen 199I) resulted in the reduction in hCG binding. The difference between the granulosa cells and the Leydig cells in the present study as regard to the effect of PRI. may indicate that the mechanism regulating LH receptors by PRL is different between the sexes. However, the possibility that the difference was due to the differences in the animals (rat, pig *vs*. mouse) and the method, such as the duration of PRL treatment (24 h *vs*. 3 days), cannot be ruled out.

Acknowledgements

The authors are grateful to Dr S Raiti, National Hormone and Pituitary Program, University of Maryland School of Medicine, NIADDK, Bethesda, MD, USA and to Dr A F Parlow, Pituitary Hormone/Antisera Center. Harbor-University of California-Los Angeles Medical Center, Los Angeles, CA, USA for the supply of pituitary hormones and antisera.

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Corresponding editor: SAMIR BHATTACHARYA